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Amino- and Carboxy-Terminal Domains of the Yeast Rab Escort Protein Are Both Required for Binding of Ypt Small G Proteins

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The Rab escort protein (REP) is an essential component of the heterotrimeric enzyme Rab geranylgeranyl transferase that modifies the carboxy-terminal cysteines of the Ras-like small G proteins belonging to the Rab/Ypt family. Deletions in the human CHM locus, encoding one of the two REPs known in humans, result in a retinal degenerative syndrome called choroideremia. The only known yeast homologue of the choroideremia gene product is encoded by an essential gene called MRS6. Besides three structurally conserved regions (SCRs) previously detected in the amino-terminal half of REPs and RabGDIs, three other regions in the carboxy-terminal domain (RCR 1–3) are here identified as being characteristic of REPs alone. We have performed the first mutational analysis of a REP protein to experimentally define the regions functionally important for Rab/Ypt protein binding, making use of the genetic system of the yeast *Saccharomyces cerevisiae*. This analysis has shown that the SCRs are necessary but not sufficient for Ypt1p binding by the yeast REP, the carboxy-terminal region also being required.

INTRODUCTION

The Rab geranylgeranyl transferase (RabGGTase)[1] is a prenyltransferase that is able to specifically recognize the carboxy-terminal cysteine motifs of the Rab/Ypt family of Ras-like small G proteins.

The Rab/Ypt-type of small G proteins plays an essential role in vesicular trafficking with a mechanism that has been highly conserved throughout evolution (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Lian et *al.*, 1994). Like other Ras-like small G proteins, they undergo conformational changes depending on their GTP- or GDP-bound status, cycling between the cytosol and a membrane-bound state (Araki *et al.*, 1991; Takai *et al.*, 1992). The Rab/Ypt small G proteins are thought either to act as specificity determinants or to ensure the progression of vesicular transport, possibly acting at more than a single transport step (Brennwald and Novick, 1993; Dunn *et al.*, 1993; Jedd *et al.*, 1995). The RabGGTase attaches geranylgeranyl lipid moieties via a thioether linkage to the terminal cysteines of the Rab/Ypt proteins. The RabGGTase is composed of two components, A and B. Component B comprises two polypeptides and is inactive without component A (Seabra *et al.*, 1992). According to the current model (Andres *et al.*, 1993; Alexandrov *et al.*, 1994), component A forms a cytosolic complex with an unmodified Rab/Ypt protein and presents it to the catalytic core of the enzyme (component B). After prenylation of the Rab/Ypt protein, component A remains bound to the geranylgeranylated protein and escorts it to the membrane; therefore, this subunit has been renamed the Rab escort protein (REP).

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[1] Abbreviations used: CHM, choroideremia; CHML, choroideremia-like; GDI, GDP-dissociation inhibitor; GDP, guanine nucleotide diphosphate; GGPP, geranylgeranyl pyrophosphate; GTP, guanine nucleotide triphosphate; RabGGTase, Rab geranylgeranyl transferase; RCR, REP-conserved region; REP, Rab escort protein; SCR, structurally conserved region; WT, wild-type.

Both in humans and in yeast, prenylation is essential for the membrane attachment and proper recycling of the Rab/Ypt-type of small G proteins from membranes (Araki *et al.*, 1991; Magee and Newman, 1992). In fact, only the prenylated forms can bind to the membrane and can be recognized by the RabGDP-dissociation inhibitor (RabGDI). The RabGDIs are a family of proteins that are able to keep the Rab/Ypt proteins in their inactive state (GDP-bound state) and to recycle them from the membrane for further rounds of association (Ullrich *et al.*, 1994; Garrett *et al.*, 1994).

The mammalian Rab escort protein REP1, encoded by the CHM locus, shares several features with RabGDIs, such as the ability to recognize and accompany the Rab proteins to the membrane and to keep them in a GDP-bound state. Similar to the RabGDIs, REP1 is also able to extract the Rab/Ypt proteins from the membrane (Alexandrov *et al.*, 1994). Therefore, according to the current model based on the mammalian system, REPs and RabGDIs differ only because the latter cannot recognize unprenylated Rab proteins or participate in Rab prenylation.

No data are yet available on the cycling of REP and Ypt proteins in yeast. The yeast counterpart of the mammalian REPs, the Mrs6 protein (Mrs6p), has been shown only to be an essential component of the yeast RabGGTase (Benito-Moreno *et al.*, 1994; Jiang and Ferro-Novick, 1994) and to be required for yeast viability (Fujimura *et al.*, 1994; Ragnini *et al.*, 1994). Interestingly, overexpression of the Mrs6 protein can partially suppress a number of different phenotypes in yeast: 1) the thermosensitive phenotype (*ts*) of the $ypt1^{N121I/A161V}$ mutant, but not the absence of Ypt1 protein (Ypt1p; Ragnini *et al.*, 1994); 2) the *ts* phenotype attributable to a mutation in the yeast Ras GTPase-activation protein (GAP) encoded by the IRA1 gene and defects in the cAMP-dependent protein kinase regulatory subunit encoded by BCY1 (Fujimura *et al.*, 1994); and 3) the respiratory deficient mutant *mrs2-1/wo* (Waldherr *et al.*, 1993a; Ragnini *et al.*, 1994).

In humans, deletions in the chromosomal locus coding for REP1 (the CHM locus) result in a syndrome called choroideremia (Cremers *et al.*, 1990; Merry *et al.*, 1992; Seabra *et al.*, 1992), an X-linked progressive tapetochoroidal dystrophy resulting in blindness in early adulthood (McCulloch, 1988). An autosomal homologue (CHML) of the choroideremia gene is located on human chromosome 1q, and its gene product REP2 has been shown to have functions overlapping those of REP1 (Cremers *et al.*, 1992; Seabra *et al.*, 1992; van Bokhoven *et al.*, 1994a,c) but with different activity toward the Rab3 subfamily (Seabra *et al.*, 1993; Cremers *et al.*, 1994) and toward the Rab27 protein (Seabra *et al.*, 1995).

We used the yeast REP Mrs6p as a model system to perform a mutational analysis on the structurally conserved regions (SCRs) shared between Rab escort proteins and RabGDIs (Boguski and McCormick, 1993; Waldherr *et al.*, 1993a) to gain information on the domain structure of this class of proteins. We show that the SCRs do contain determinants for Ypt protein binding, but they are not sufficient for this function.

Studies on the mutational spectrum of choroideremia patients have shown that choroideremia patients carry deletions of the whole reading frame or mutations leading to REP1 carboxy-terminal truncations (van den Hurk *et al.*, 1992; Donnelly *et al.*, 1994; van Bokhoven *et al.*, 1994b). Here we show that carboxy-terminal truncation of the Mrs6 protein affects RabGGTase activity because of the inability of the mutant protein to bind its substrates, the small G proteins.

MATERIALS AND METHODS

Strains and Media

Escherichia coli XL1-blue (Stratagene, La Jolla, CA), JM109, and BMH71-18 mutS (Promega, Madison, WI) were used for plasmid amplification and propagation. The following *Saccharomyces cerevisiae* strains were used: AR2 (MATa/α, MRS6/*mrs6*::HIS3, *leu2/leu2*, *ura3/ura3*, *his3/his3*, *trp1/trp1*, *ade8/ade8*, rho^+ mit^+; Ragnini *et al.*, 1994); AR4-6B (MATa, *ura3*::Gal1/10-MRS6-URA3, *mrs6*::HIS3, *leu2*, *his3*, *trp1*, *ade8*, rho^+ mit^+; Benito-Moreno *et al.*, 1994); and Y190 (MATa, *gal4*, *gal80*, *ade2-101*, *his3*, *trp1-901*, *ura3-52*, *leu2-3,-122*-URA3::Gal-lacZ, *lys2*::Gal-HIS3, cyh^r; Harper *et al.*, 1993). The following media were used: LB, TYP (Sambrook *et al.*, 1989); YPD, YP-Gal, SD, SPO (Ragnini *et al.*, 1994); S-Gal (0.67% Difco yeast nitrogen base, 2% galactose) supplemented with bases and amino acids as required.

Plasmid Constructs

The plasmids YEpM6PN and YCplacM6PN (expressing the wild-type [WT] Mrs6p) and the plasmid YEpM6PNS were obtained as previously described (Waldherr *et al.*, 1993a; Ragnini *et al.*, 1994). The YEpM6ΔNhe and YEpM6XP plasmids were constructed by digesting YEpM6PN with *Nhe*I-*Pst*I or with *Xba*I-*Pst*I, respectively, followed by religation. The resulting MRS6 ORFs are fused at position +1711 and +1290, respectively, with the plasmid polylinker. So that the YEpM6ΔXba and the YCpM6ΔXba constructs could be obtained, YEpM6XP was digested with *Kpn*I-*Sph*I and recloned in the vector YEplac181 or YCplac33, respectively. A *Kpn*I-*Sac*I 2.28-kb M6ΔNhe fragment from YEpM6ΔNhe was cloned in the YCplac33 vector to obtain the YCpM6ΔNhe construct. The YEpM6ΔNhe and the YEpM6ΔXba plasmids express Mrs6 mutant proteins, named $Mrs6^{\Delta Nhe}$ and $Mrs6^{\Delta Xba}$, respectively, that have 570 and 429 amino acids from the Mrs6 protein plus 14 and 7 amino acids, respectively, derived from the translation of the plasmid polylinker. The plasmids used for the two-hybrid analyses were obtained as follows. M6Δ*Bam*HIpAS2: a 981-bp fragment of the MRS6 ORF obtained by polymerase chain reaction (PCR) using the oligo Mrs6NdeI (5′ACTAATATTGACCGAAAAAAACATATGTTAAGT3′) and the oligo Mrs6BamHI (5′CTTCAACTTTGGATCCATCTTGGAATG3′) was cloned in the *Nde*I-*Bam*HI sites of the plasmid pAS2. M6PNSpAS2: an 810-bp *Bam*HI-*Sal*I fragment from the plasmid YEpM6PNS was digested and cloned in the same sites of the M6Δ*Bam*HIpAS2 construct (Ragnini *et al.*, 1994). Therefore, the M6PNSpAS2 construct contains a truncated Mrs6 ORF at position +1783. M6PNSpACTII: a 1.8-kbp *Nde*I-*Sal*I fragment of M6PNSpAS2 was cloned in the *Sma*I-*Xho*I sites of the plasmid pACTII. M6ΔXbapACTII: a 1.3-kbp *Nde*I-*Xba*I fragment from the plasmid M6PNSpAS2 was isolated, blunt-ended, and cloned in the pACTII digested with *Sma*I. The correct orientation was determined

by restriction analysis. M6ΔNhepACTII: a 1.7-kbp *Nde*I-*Nhe*I fragment from the M6NXpET22 plasmid (Benito-Moreno *et al.*, 1994.) was digested, blunt-ended, and cloned into the *Sma*I site of pACTII vector. The correct orientation was checked by restriction analyses. YPT1 pAS2 and pACTII constructs: a 0.95-kb *Nde*I-*Bam*HI fragment from plasmid YPT1pLN (kindly provided by Dr. H.D. Schmitt, MPI, Göttingen, Germany) was cloned into the same sites of the vector pAS2 (YPT1pAS2) or was blunt-ended at the *Nde*I site and cloned in the *Sma*I-*Bam*HI sites of pACTII (YPT1pACTII).

Site-directed Mutagenesis

Altered Sites in vitro Mutagenesis System (Promega), as described by the manufacturer, was used. A 3.23-kb *Sac*I-*Pst*I fragment from YEpM6PN containing the full-length Mrs6 ORF was cloned into the *Sac*I-*Pst*I sites of the pAlter-1 vector. The oligonucleotides $Mrs6^{G53E}$ 5′GTTTTAATCGCAGAGACTGGTATGGTA3′; $Mrs6^{H74D}$ 5′TCCAATGTCCTTGATATTGATAAGAAT3′; $Mrs6^{D132H}$ 5′GATTTCGGTATTCATCTTTCCCCGAAG3′; and $Mrs6^{Q289E}$ 5′GGAGAATTGTCGGAAGGGTTTTGTAGA3′ were used. After mutagenesis, the recombinant plasmids were screened directly by sequencing. The mutated MRS6 genes were then recloned in the *Sac*I-*Pst*I sites of high- (YEp352, YEplac181, YEp351; Hill *et al.*, 1986) or low-copy plasmids (YCplac33; Gietz and Sugino, 1988).

Determination of Metabolic Activity

Cells were incubated in 0.025% trypan blue for 5 min at room temperature. The percentage of white cells was then determined.

Two-Hybrid Analyses

Two-hybrid analyses were performed with the system developed and kindly supplied by Dr. S.J. Elledge (Baylor College of Medicine, Houston, TX). LEU^+ TRP^+ transformants were selected after cotransformation of the strain Y190 with either YPT1pACTII and the MRS6 mutant versions cloned in the pAS2 construct or YPT1pAS2 and the different MRS6-pACTII derivatives. The colonies producing β-galactosidase were identified either by a filter assay or by crude extract assay as described (Amberg *et al.*, 1995). For negative controls, the single pACTII- or pAS2-derivative constructs were transformed in the strain Y190. Positive interactions were checked by plasmid loss, after which β-galactosidase activity was determined again.

Mrs6 and Ypt1 Protein Expression, Purification, and Antibody Production

An *Nde*I (position +1) and *Xho*I (position +1801) were created in the MRS6 ORF by using the Mrs6NdeI oligo (see above, Plasmid Constructs) and the Mrs6XhoI oligo (5′TCATATCTCCTCTTGAGTTCACCTACAAATTCACCGGAACC3′). After PCR amplification, the 1.8-kb *Nde*I-*Xho*I MRS6 fragment was cloned in the pET22b vector from Novagen (M6NXpET22b construct; Madison, WI). The 6xHis-tagged Mrs6p was expressed and purified from *E. coli* extracts by native purification on Ni^{2+}-agarose as described by the manufacturer (Qiagen, Hilden, Germany), followed by concentration and desalting by a Centricon-10 (Amicon, Beverly, MA). The anti-Mrs6p polyclonal antibody, named SLM6-1 Ab, was raised in rabbits, and the serum was purified on Protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. The SLM6-1 Ab specificity was determined by immunoblot analyses, as described in the text. To detect the $Mrs6^{\Delta Xba}$ protein after coimmunoprecipitation, we used a second anti-Mrs6 antibody, named BMM6-1, derived from the 1.15-kb *Ssp*I-*Nhe*I central fragment of the MRS6 gene (Benito-Moreno *et al.*, 1994). The Ypt1p was fused with a 6xHis-tag at its NH_2 terminus by cloning an *Nde*I-*Bam*HI YPT1-containing fragment from plasmid Ypt1pLN (kindly given by Dr. H.D. Schmitt) in the *Nde*I-*Bam*HI sites of the vector pET15b (Novagen). The Ypt1p was purified from *E. coli* extracts under native conditions on Ni^{2+}-agarose (Qiagen), as described by the manufacturers. The structural integrity of the purified 6xHis-Ypt1p and the ability to bind GTP/GDP was tested by a GTP-binding assay on a nitrocellulose filter as described by Schmitt *et al.*, 1986. The ability of the His-tagged Ypt1p to be prenylated by the WT yeast RabGGTase was tested by geranylgeranylation assay with WT yeast extracts. The anti-Ypt1 mouse monoclonal antibody was a gift from Dr. D. Gallwitz (MPI, Göttingen, Germany).

Yeast Transformation, Protein Extracts, Western Blots, and Immunodetections

Yeast transformation was performed with the LiAC method as described by Soni *et al.*, 1993. The AR4-6B strain was transformed with the YEp-Mrs6 derivatives, and transformants were selected in SD or S-Gal media supplemented with amino acids and bases as appropriate. To shut off the galactose-regulated expression of the ectopically integrated copy of MRS6 gene in AR4-6B cells transformed with the YEpM6PNS, with the inviable Mrs6p constructs (YEpM6G53E, YEpM6ΔXba), or with the empty vector (YEp351), we precultured cells in S-Gal Leu^- medium until late exponential phase (OD_{600} = 1.5), washed them, and diluted them to OD_{600} = 0.05 in 150-ml YPD medium. Cells were then harvested at different time points from the shift to the nonpermissive conditions. In the cases of the complementing Mrs6 constructs YEpM6PN (WT Mrs6p), YEpM6H74D, YEpM6D132H, YEpM6Q289E, and YEpM6ΔNhe, cells were selected directly on SD Ura^- plates and then precultured for 12 h in the same medium, washed, diluted to OD_{600} = 0.05 in 150 ml of YPD, and grown for 6 h in YPD liquid media. After harvesting, the cells were resuspended in 500 μl of lysis buffer (20% glycerol, 100 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). An equal volume of glass beads was added, and lysates were obtained after 45 min of vigorous shaking at 4°C on an IKA-VIBRAX-VXR shaker (Janke & Kunkel, GmbH & Co., Staufen, Germany). Samples were then centrifuged at 5000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). Equal amounts of protein (10–20 μg) were electrophoresed on SDS-PAGE and transferred onto nitrocellulose paper (BA85; Schleicher & Schuell, Keene, NH) by semidry blotting using a SEMIPHOR TE 70 electroblotter (Hoefer, San Francisco, CA). Immunological detection was performed as recommended by the ECL Western blotting protocols (Amersham, Arlington Heights, IL) with 5% nonfat dry milk as a blocking agent. The anti-Mrs6p rabbit polyclonal antibodies SLM6-1 and BMM6-1 were used at a dilution of 1:10,000 and 1:150, respectively; the anti-Ypt1p monoclonal antibody at 1:150; the anti-rabbit (Promega) and anti-mouse (Pierce, Rockford, IL) immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibodies were used at a dilution of 1:12,000. The extracts that did not contain Mrs6p expressed from the Gal1/10-regulated promoter, as determined by immunodetection with anti-Mrs6 antibody, were then used for geranylgeranylation assays and for the coimmunoprecipitation experiments.

Geranylgeranylation Assay

The geranylgeranylation assay was performed essentially as described previously (Benito-Moreno *et al.*, 1994), except that 250 μg of yeast crude extract was used in a final volume of 60 μl with or without addition of 10 μg of 6xHis-Ypt1 protein and/or 6 μg of 6xHis-Mrs6p, as indicated in the text. In all, 15 μl aliquots were electrophoresed on 10% Tricine-SDS gels. After fluorography and drying, the gel was exposed to an X-Omat AR Kodak film for 1 wk. Signals quantification was performed by densitometric scanning of the autoradiograms with the BIO-PROFIL package BIO-1D software version 6.02c (Biotechnology Division, Vilber Lourmat, Marne-La-Vallee, France).

Coimmunoprecipitation Assay

Total protein extract (1 mg) was incubated for 3 h at 4°C with 1 μl of purified rabbit anti-Mrs6p polyclonal antibody SLM6-1 either with or without the simultaneous addition of 2 μg of 6xHis-YPT1p, as described in the text. After incubation, the mixture was then subjected to precipitation on protein A-Sepharose CL-4B beads (50 μl of a 10% slurry/μl of antibody). The beads were washed three times with 1 ml of immunoprecipitation buffer (50 mM potassium phosphate, pH 7.6, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.014% Triton X-100, and 0.01% NP40) and then solubilized in 30 μl of 2× SDS-loading buffer. After boiling, one-fifth of the mixture was loaded on a 12.5% SDS-PAGE. Western blotting and immunodetection were performed as described above with anti-Mrs6p rabbit polyclonal (SLM6-1 Ab) and/or anti-Ypt1p mouse monoclonal antibodies. Because of the comigration in the immunoprecipitable fraction of the $Mrs6^{\Delta Xba}$ protein with the IgG (running as 45–48 kDa), the Western blots carrying this protein were stripped and reprobed (following the ECL manufacturer's instructions) with the anti-Mrs6 antibody BMM6-1 (see Figures 6B and 7C).

RESULTS

Mutational Analyses of the Mrs6 Protein

We have suggested previously that the structurally conserved regions (SCRs) that we identified in the amino-terminal part of the REP/RabGDI proteins could contain the structural requirements for Rab/Ypt binding (Waldherr *et al.*, 1993b). To test this hypothesis, we performed site-directed mutagenesis on some of the most conserved amino acids located in the SCR1A, SCR1B, SCR2, and SCR3B. The amino acids to be changed were chosen in such a way that neither the predicted secondary structure nor the hydrophobicity profile of the Mrs6 protein would have major changes, as calculated by the programs NOVOTNY, GARNIER, and GGBSM of the PCgene package version 6.26, while the local charge would be affected.

For this purpose, we replaced glycine 53 with a glutamic acid residue in SCR1a (mutation G53E); in the SCR1b box we replaced histidine 74 with an aspartic acid residue (mutation H74D); in the SCR2 box we replaced aspartic acid 132 with histidine (mutation D132H); and in SCR3b we replaced glutamine 289 with a glutamic acid residue (mutation Q289E). A schematic diagram of Mrs6p is shown in Figure 1A, along with the positions of mutations generated in its sequence.

A further search for additional domains shared only by the REPs (Figure 1C) was performed with the multiple alignment program MACAW 2.0.1 (Blosum 62). Three blocks with significant homology (calculated by MACAW according to Karlin and Altschul, 1990) were found at the carboxy terminus of the REPs (Figure 1C), which we have called REP-conserved regions (RCR1, aa 478–494; RCR2, aa 522–555; RCR3, 555–570; Mrs6p, National Center for Biotechnology Information [NCBI] 585776). The second and third of these motifs overlap in the Mrs6 protein while they are divided by a proline-rich stretch of 9 amino acids in the mammalian REPs. We have observed that all three carboxy-terminal motifs are deleted in patients having the shortest deletions in the CHM locus (patient CN: van Bokhoven *et al.*, 1994b; patients CHM 2086, CHM 17.1, CHM 2084: van den Hurk *et al.*, 1992), indicating that this carboxy-terminal region is important for RabGG-Tase activity.

We created carboxy-terminal deletions of Mrs6p either to retain the RCR motifs by deleting the last 33 amino acids (mutation ΔNhe; Figure 1) or to eliminate them by deleting the last 174 amino acids (mutation ΔXba; Figure 1). This second deletion point was chosen because alignment of the the REP1-deleted proteins of choroideremia patients and the Mrs6 protein with respect to the SCRs shows that this deletion point would correspond to that of the choroideremia patient CHM 2086 (van den Hurk *et al.*, 1992). The studies on the $Mrs6^{\Delta Xba}$ protein will therefore provide us with in vivo and in vitro experimental data on the effects that such carboxy-terminal deletions could have on REP activity.

In this study we have also analyzed the effects on RabGGTase activity and Ypt1p-binding of a previously described 9-amino-acid carboxy terminally deleted (mutation PNS; Figure 1) Mrs6 protein (Waldherr *et al.*, 1993a; Ragnini *et al.*, 1994). This MRS6 carboxy-deleted construct (YEpM6PNS) was found during the screening of high-copy-number suppressors of the respiratory-deficient mutant *mrs2-1/wo*. We have shown previously that the plasmid expressing this mutant version of the Mrs6 protein is able to complement Mrs6p-depleted cells (Ragnini *et al.*, 1994) as well as the thermosensitive mutant $Ypt1^{N121/A161V}$ (A. Ragnini, unpublished results).

Functional Analysis of the Mutant Mrs6 Proteins

To perform complementation tests with the mutant Mrs6 proteins, we generated a strain (AR4-6B) that conditionally expresses a galactose-regulated chromosomal copy of the MRS6 gene (Benito-Moreno *et al.*, 1994). Upon a shift from galactose- to glucose-containing media, the cells are depleted of Mrs6p within 10–12 h, stop growing within 14–18 h, and die later on, as determined by trypan blue staining. Transformation with plasmids carrying the MRS6 gene or the mutant constructs under the control of the MRS6 promoter before the shift allowed us to test whether these gene copies can support growth in glucose-containing media. Wild-type and mutated MRS6 gene constructs (cloned in low-copy-number vectors) were also used to transform the diploid strain AR2 that has one of the MRS6 gene copies disrupted (MRS6/*mrs6*::HIS3; Benito-Moreno *et al.*, 1994), and the transformants were subjected to sporulation and tetrad analysis. Growth of four spores per tetrad indicated that the mutant

MRS6 construct was still functionally active and could substitute for the mrs6-disrupted copy.

According to these tests, two mutant proteins were found not to be able to support growth of yeast cells, namely the $Mrs6^{G53E}$ protein (mutated in SCR1a) and the $Mrs6^{DXba}$ carboxy-terminal–deleted protein (−174 amino acids). All the other Mrs6 mutant proteins ($Mrs6^{H74D}$, $Mrs6^{D132H}$, $Mrs6^{Q289E}$, $Mrs6^{\Delta Nhe}$, and $Mrs6^{PNS}$) were sufficiently functional to sustain growth of yeast cells, as summarized in Figure 1B. A typical growth curve obtained from the strains transformed with the inviable constructs, the WT Mrs6p (wt), the carboxy-terminal–deleted PNS constructs, or the empty YEp vector (negative control) is shown in Figure 2.

RabGGTase Activity Is Abolished by a Mutation in SCR1a and by a Carboxy-Terminal Deletion in Mrs6p

We then tested the ability of yeast crude extracts from the AR4-6B strain expressing the complementing ($Mrs6^{H74D}$, $Mrs6^{D132H}$, $Mrs6^{Q289E}$, $Mrs6^{\Delta Nhe}$, and $Mrs6^{PNS}$) or the noncomplementing ($Mrs6^{G53E}$ and $Mrs6^{\Delta ba}$) Mrs6 protein versions to sustain prenylation of an *E. coli*-expressed His-tagged Ypt1 protein (His-Ypt1p) by an in vitro geranylgeranylation assay.

The extracts containing the complementing Mrs6 mutant proteins were prepared from AR4-6B transformants grown in glucose-containing media. The crude extracts from cells expressing the noncomplementing mutant proteins ($Mrs6^{G53E}$ and $Mrs6^{\Delta Xba}$) and the complementing $Mrs6^{PNS}$ protein were prepared from cells precultured in galactose-containing media and then shifted to glucose-containing media at a time when no more WT Mrs6p could be detected by immunoblot analyses (the ectopically integrated MRS6 gene expresses a protein that can be distinguished from the plasmid-encoded copy because it migrates 6 kDa above the latter; Benito-Moreno *et al.*, 1994) and when the metabolic activity of the noncomplementing mutants was above 80% (usually 18 h after the shift to

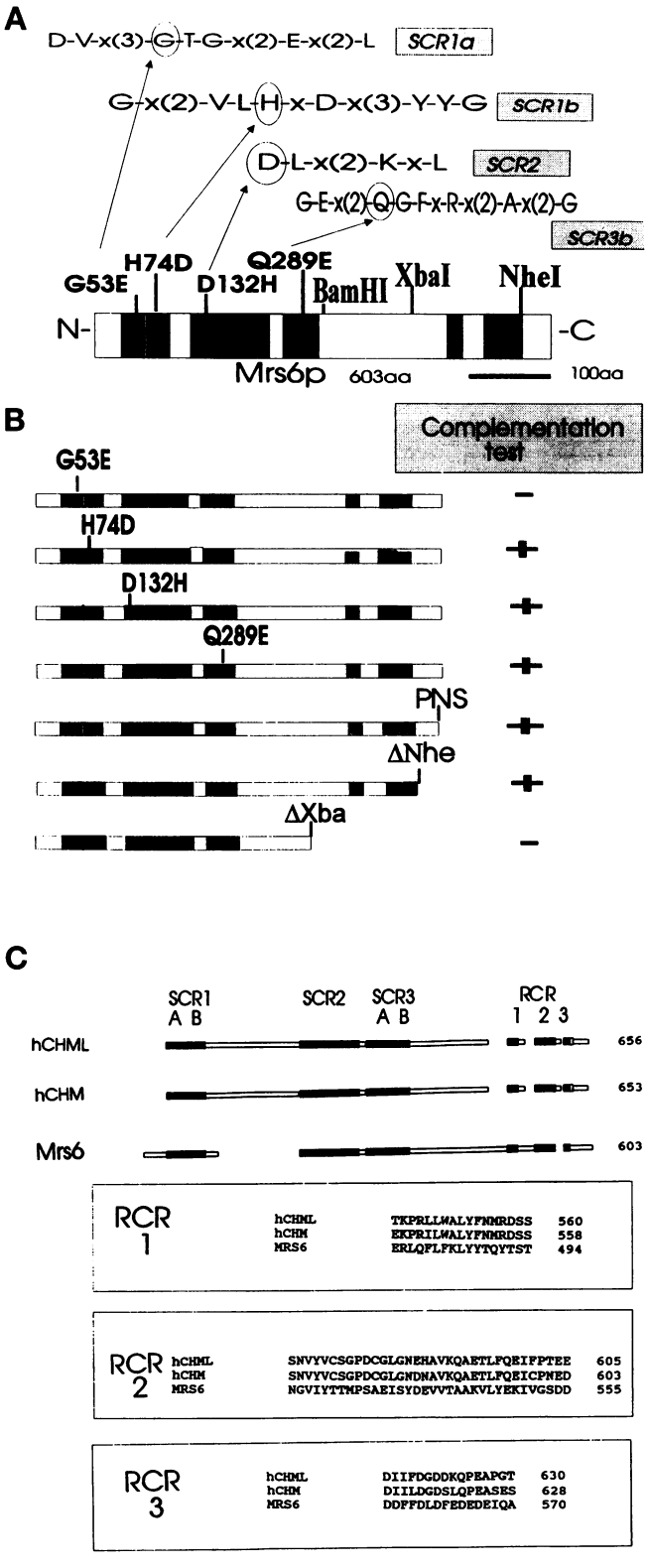

Figure 1. Schematic diagram of conserved domains in the Mrs6 protein. (A) Domains conserved in REP and RabGDI proteins (SCR 1–3, black boxes) and domains unique to REPs (RCR 1–3, gray boxes). The SCR motifs are given in PROSITE notation (Waldherr *et al.*, 1993b) with amino acids in the single-letter code and X indicating any residue. Amino acid exchanges introduced in this work are circled. (B) Mutant Mrs6 proteins and their ability to complement Mrs6p-depleted cells. PNS, ΔNhe, and ΔXba are Mrs6p carboxy-terminal deletions of 9, 33, and 174 amino acids, respectively. Complementation tests were performed as described in the text; + indicates complementation of Mrs6p-depleted cells, and − indicates lack of complementation. (C) Comparison of Rab escort proteins. Multiple alignment of Rab escort proteins from human (hCHM, NCBI 460795; hCHML, NCBI 116365) and yeast (MRS6, NCBI 585776; Ragnini *et al.*, 1994). The multiple alignment was performed with the program Macaw 2.0.1 using Blosum 62. Conserved blocks **(Figure 1 cont.)** are shown as black boxes (SCRs, Waldherr *et al.*, 1993b) or gray boxes (RCRs). The numbers given at the end of each sequence correspond to the length of the respective proteins.

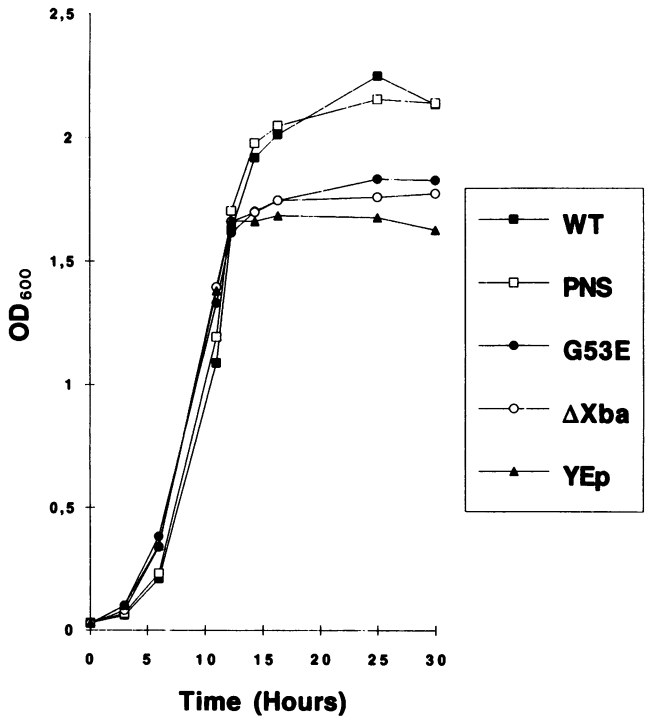

Figure 2. Growth curves of AR4-6B cells transformed with the WT MRS6, PNS, or YEp 351 constructs or with the indicated noncomplementing constructs (carried on YEp181 or YEp351 vectors) after the shift from galactose- to glucose-containing media. Optical density (OD) was measured at 600 nm.

the nonpermissive carbon source). Under these conditions, all crude extracts contained approximately equal amounts of the respective Mrs6 plasmid-encoded proteins as measured by immunoblot analysis (Figure 3).

As an isoprenyl-group acceptor for the in vitro geranylgeranylation assay, we have used bacterially expressed His-Ypt1p, the structural integrity of which was tested by measuring its ability to bind $^{[32P]}$GTP in a GTP-overlay assay and by its ability to be properly recognized by WT RabGGTase (as described in MATERIALS AND METHODS).

Extracts obtained from cells expressing the WT Mrs6 protein (WT extracts) or the complementing Mrs6 mutant proteins Mrs6^{H74D}, Mrs6^{D132H}, Mrs6^{Q289E}, and Mrs6$^{\Delta Nhe}$ were all found to have detectable RabGGTase activity (Figure 4A). However, clearly reduced activity was detected in the extract containing the Mrs6^{H74D} protein and, to a much greater extent, in the extract containing the Mrs6^{D132H} protein. To estimate the relative amounts of $^{[3H]}$GGPP transferred to the His-Ypt1p substrate, we performed quantification of the autoradiograms by densitometric scanning of geranylgeranylation assays. The average values obtained for each mutant, considering the amount of His-Ypt1p geranylgeranylated by the WT extracts as 100%, is given in Figure 4B. The results show that the Mrs6^{D132H} mutant protein has ~20% of the WT activity, whereas the Mrs6^{H74D}, Mrs6^{Q289E}, and Mrs6$^{\Delta Nhe}$ mutant proteins have ~68, 78, and 77%, respectively.

Figure 5 shows the autoradiogram obtained from the geranylgeranylation assay using the crude extracts containing the noncomplementing Mrs6^{G53E} and Mrs6$^{\Delta Xba}$ proteins and the complementing Mrs6^{PNS} protein. Although RabGGTase activity can be detected at time 0 (which in any case includes the activity of the Gal1/10-MRS6-encoded protein; Figure 5, left panel), no activity could be detected 18 h after the shift in the extracts containing either the Mrs6^{G53E} or the Mrs6$^{\Delta Xba}$ proteins nor in extracts that did not contain any Mrs6p (the AR4-6B strain transformed with the YEp351 empty vector; *lane* YEp in Figure 5). Addition of *E. coli*-purified Mrs6p to these extracts restored RabGGTase activity as shown, for example, in Figure 5 (*lane* Mrs6p, right panel) for cells that did not express any Mrs6p. As expected, normal RabGGTase activity (>90% when compared with the WT prenylation activity) was detected in the extract containing the Mrs6^{PNS} protein (*lane* PNS, Figure 5, right panel).

These results clearly show that 1) the mutation G53E (in SCR1a) or the deletion of 174 amino acids from the carboxy terminus of the Mrs6p (Mrs6$^{\Delta Xba}$ protein) completely abolish RabGGTase activity; 2) the H74D and D132H mutations (in SCR1b and SCR2, respectively) result in a reduced RabGGTase activity; 3) the Q289E mutation (SCR3b) or the deletion of the last 33 (ΔNhe) or 9 amino acids (PNS) of the Mrs6p has only minor effects on the RabGGTase activity.

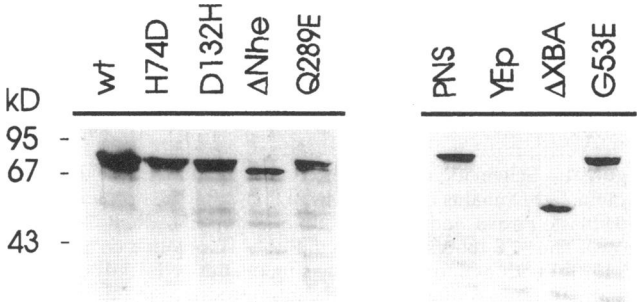

Figure 3. Specificity of the anti-Mrs6p antibody SLM6-1 and stability of the Mrs6 mutant proteins, as determined by immunoblot analysis. Extracts from the AR4-6B cells transformed with the empty vector YEp351, wild-type MRS6 gene (wt), and the indicated mutant MRS6 constructs carried on YEp vectors were prepared after 18 h from the shift to glucose-containing media. For each sample, 30 μg was separated by 12.5% SDS-PAGE before transfer to nitrocellulose. After incubation with the primary anti-Mrs6 and the secondary antibodies, detection was performed by the ECL method.

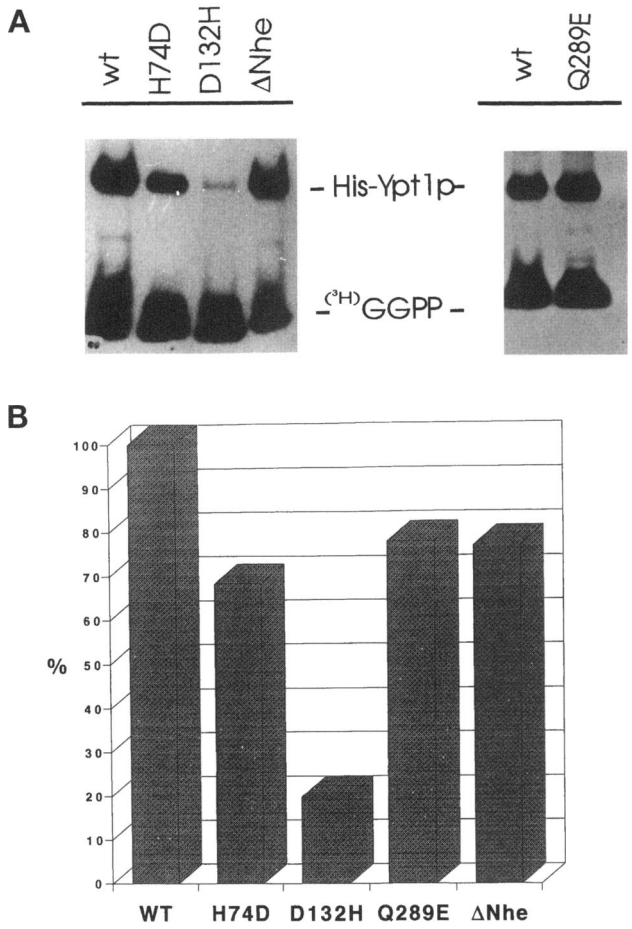

Figure 4. Geranylgeranylation assays using crude extracts from the AR4-6B cells grown in glucose-containing media after transformation with YEpM6PN (wt), YEpM6H74D, YEpM6D132H, YEpM6ΔNhe, and YEpM6Q289E. (A) Reactions were performed with the addition of 10 μg of His-Ypt1p (in absence of bacterially expressed His-Mrs6p, as described in MATERIALS AND METHODS). Labeled His-Ypt1p and unincorporated (^{3}H)GGPP are indicated. (B) Quantification of His-Ypt1p prenylation shown as the average of three independent experiments (the highest SD value, ±16%, was calculated for H74D; all the others were below ± 12%). The amounts of prenylated His-Ypt1p are indicated in percentages (%) relative to the wild-type extracts, which were taken as 100%.

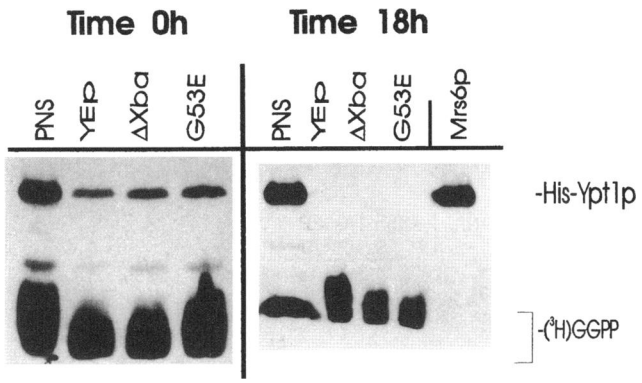

Figure 5. Geranylgeranylation assays using crude extracts from the strain AR4-6B transformed with YEpM6PNS, YEpM6G53E, and YEpM6ΔXba, or the YEp351 empty vector. Cells were grown in galactose-containing media and then shifted to glucose-containing media. Yeast extracts were prepared from samples taken at time 0 (left panel) or at time 18 h (right panel) after the shift (see MATERIALS AND METHODS). His-Ypt1p (10 μg) was added as substrate. In the lane marked Mrs6p, the reaction was performed as above with the 18-h crude extract from cells transformed with YEp351 to which 6 μg His-Mrs6p was added. Labeled His-Ypt1p and unincorporated (^{3}H)GGPP are indicated.

SCRs and the Mrs6p Carboxy-Terminal Regions Are Required for Mrs6p–Ypt1p Binding

To further characterize the nature of the RabGGTase defects caused by the mutations created in Mrs6p, we tested whether they could be attributed to a reduced ability to bind Ypt proteins. Purified anti-Mrs6p antibody was used to coimmunoprecipitate endogenous Mrs6–Ypt1 protein complexes from crude extracts containing the different mutant versions of Mrs6p. The precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal anti-Ypt1p antibody (bottom panels in Figures 6, A and B, and 7, A–C) and anti-Mrs6p antibody (top panels in Figures 6, A and B, and 7, A–C). Endogenous Ypt1p was coimmunoprecipitated from all extracts bearing the complementing mutations H74D, D132H, Q289E, ΔNhe (Figure 6A), and PNS (Figure 6B) or

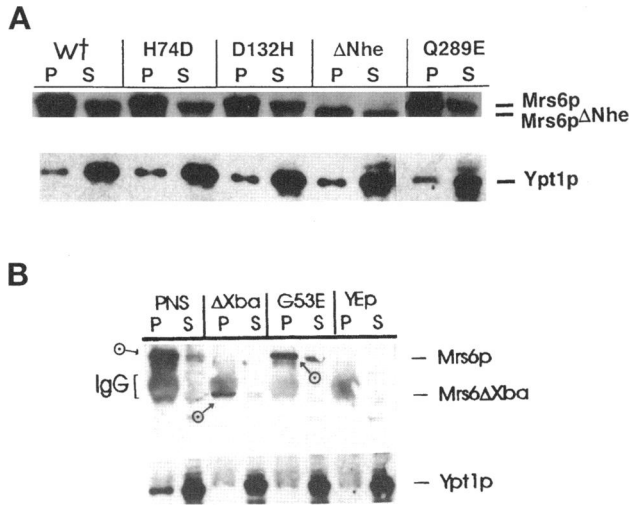

Figure 6. . Coimmunoprecipitation of the Ypt1p with the wild-type and mutant Mrs6 proteins. The extracts from AR4-6B cells expressing the mutated or WT Mrs6 proteins were immunoprecipitated with the anti-Mrs6p antibody. The pellet (lanes P) and the nonprecipitable material (lanes S) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose. ECL detection was performed after incubation with the anti-Mrs6 antibody (A and B, top panel) or with the anti-Ypt1p antibody (A and B, bottom panel). (B) In lanes P, the positions of full-length Mrs6, Mrs6^{G53E}, and Mrs6$^{\Delta Xba}$ proteins are indicated by arrows.

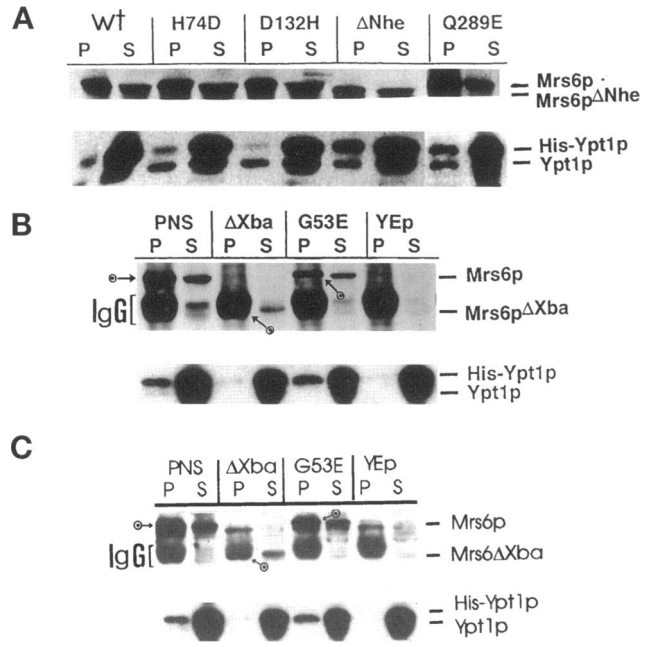

Figure 7. Coimmunoprecipitation of wild-type Ypt1p and/or His-Ypt1p with wild-type and mutant Mrs6 proteins. *E. coli*-purified His-Ypt1p was added, together with the anti-Mrs6p antibody, to the same crude extracts used in Figure 6. The pellet (lanes P) and the nonprecipitable material (lanes S) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose. ECL detection was performed after incubation with the anti-Mrs6p antibody SLM6-1 (A and B, top panel) or BMM6-1 (C, top panel) or with the anti-Ypt1p antibody (A–C, bottom panel). (B and C) In lanes P, the positions of full-length Mrs6, $Mrs6^{G53E}$, and $Mrs6^{\Delta Xba}$ proteins are indicated by arrows.

containing the WT Mrs6p (Figure 6A). By contrast, no Ypt1p could be detected in the immunoprecipitates from crude extracts prepared from cells expressing the noncomplementing mutations G53E and DXba or from Mrs6p-depleted cells (lane YEp; Figure 6B). This indicates that the WT Mrs6p as well as the $Mrs6^{H74D}$, $Mrs6^{D132H}$, $Mrs6^{Q289E}$, $Mrs6^{\Delta Nhe}$, and $Mrs6^{PNS}$ proteins can be found in a complex with the endogenous Ypt1p, whereas at steady-state levels the $Mrs6^{G53E}$ and $Mrs6^{\Delta Xba}$ proteins do not form a stable complex with this protein.

To test the ability of the mutant Mrs6p to form new complexes with unprenylated Ypt1p, we added *E. coli*-expressed histidine-tagged Ypt1p (His-Ypt1p) to the crude extracts, prepared as in the previous experiment. Because the His-Ypt1p is added together with the anti-Mrs6p antibody, we expected to detect complexes formed immediately upon addition of the His-Ypt1p plus the complexes already formed with the endogenous Ypt1p.

As shown in Figure 7A, the WT Mrs6p can bind equal amounts of His-Ypt1p and endogenous Ypt1p (the latter migrates faster than the former). A similar result was also obtained with extracts containing the $Mrs6^{Q289E}$ protein. However, both the $Mrs6^{H74D}$ and $Mrs6^{D132H}$ proteins show a reduction in the amount of the His-Ypt1p complex with respect to that formed with endogenous Ypt1p, which seems to be the same as in the WT case (Figure 7A). It is possible that the altered binding ability of the $Mrs6^{H74D}$ and $Mrs6^{D132H}$ proteins with the His-Ypt1p does not reflect the situation in vivo but only the inability of these proteins to recognize this particular substrate. We therefore tested whether the RabGGTase activity of the cells expressing the $Mrs6^{H74D}$ and $Mrs6^{D132H}$ proteins was also altered in vivo by adding purified His-Mrs6p to the geranylgeranylation assay in the absence of His-Ypt1p. If, consistent with results obtained with the geranylgeranylation of the His-Ypt1p, the RabGGTase activity is reduced in vivo, Ypt proteins should accumulate unprenylated in the mutant extracts. Addition of Mrs6p to these extracts would provide the missing activity; therefore, the endogenous unprenylated proteins will be labeled by the transfer of the tritiated geranylgeranyl pyrophosphate.

For this purpose crude extracts were prepared from glucose-grown AR4-6B cells transformed with YEpM6H74D, YEpM6D132H, or YEp351 (in this case the sample was taken at 14 h from the shift from galactose- to glucose-containing media). A geranylgeranylation assay was then performed as described previously, except that no His-Ypt1p was added to the assay, and 6 μg of purified His-Mrs6p was used to reconstitute the RabGGTase activity.

As shown in Figure 8A, it is possible to label by geranylgeranylation assay proteins in the range of 24–28 kDa using extracts expressing the $Mrs6^{H74D}$ and $Mrs6^{D132H}$ proteins (panel 3) or extract lacking Mrs6p (panel 4). This result indicates that unprenylated proteins accumulate in these extracts, and, as expected, the highest accumulation is detectable in the strains that do not express any Mrs6p (YEp (+), panel 4). The reconstitution of the RabGGTase activity clearly depends on the addition of the His-Mrs6p to the mutant extracts because no (lanes D132H, panel 2; YEp (−), panel 4) or very little (lane H74D, panel 2) labeling of endogenous Ypt proteins can be detected in the absence of Mrs6p. Quantification of the signals obtained in panels 3 and 4 is shown in Figure 8B. In the $Mrs6^{H74D}$ crude extracts, only 38% of proteins in the range of 24–28 kDa can be labeled because of the His-Mrs6p addition, whereas in the $Mrs6^{D132H}$ crude extracts ~76% of proteins in the same range are labeled (considering the unprenylated proteins detected in the Mrs6p-depleted cells as 100%; lane YEp [+]). These results correlate very well with the estimated RabGGTase activity of the mutant Mrs6p (68% for $Mrs6^{H74D}$ and 20% for the $Mrs6^{D132H}$ mutant proteins; Figure 4B).

Therefore, the results obtained with the cells expressing the Mrs6^{H74D} and Mrs6^{D132H} proteins show that reduced RabGGTase activity is observed in vivo and in vitro. Also, both in vivo and in vitro a comparable reduction in the amount of complex formation between the His-Ypt1 and the mutated Mrs6 proteins is seen. However, at steady-state levels, normal amounts of complexes are formed with the endogenous Ypt1p. Although more detailed experiments on the kinetics of binding are required, one possibility might be that these mutations affect the binding/releasing constant of complex formation in vitro as well as in vivo. A slowdown in the rate of releasing of the Ypt substrate would result in a delay in prenylation that could create the observed accumulation of unprenylated Ypt without affecting the amount of complexes found at steady-state levels (see DISCUSSION).

The coimmunoprecipitation experiments performed with the PNS extract show that this protein is preferentially coimmunoprecipitated with His-Ypt1p (Figure 7B) instead of the WT Ypt1p (the latter is visible after prolonged exposure of the films), although in the absence of His-Ypt1p it is normally found in a complex with the WT Ypt1p (Figure 6B).

A much less dramatic but similar effect seems to occur in the case of the Mrs6$^{\Delta NHe}$ protein (Figure 7A) although, in this case, no dissociation of the complexes already formed with the WT Ypt1p was observed. The coimmunoprecipitation experiments performed with the extracts that contain the noncomplementing Mrs6^{G53E} and Mrs6$^{\Delta Xba}$ proteins showed that these two proteins do not interact with the endogenous Ypt1 protein (Figure 6B). However, as shown in Figure 7B, Mrs6^{G53E} protein can form a complex with His-Ypt1p, indicating that its binding activity is not completely abolished. No WT Ypt1 protein was detectable in the immunoprecipitable fraction even after prolonged exposure of the film, confirming the inability of the Mrs6^{G53E} protein to bind the WT Ypt1 protein.

A signal barely above background levels, corresponding to His-Ypt1p but not the WT Ypt1p, was observed in the immunoprecipitated fraction from extracts containing the Mrs6$^{\Delta Xba}$ protein (lane ΔXba in Figure 7C). This result confirmed that the deletion of the last 174 amino acids of Mrs6p severely reduces or abolishes Ypt1p binding and that this part of the protein contains important determinants for Mrs6p–Ypt1p binding.

Taken together, the results obtained with the coimmunoprecipitation experiments show that the mutations G53E, H74D, and D132H located in SCR1a, SCR1b, and SCR2, respectively, affect Ypt1p binding, but the strongest effect is obtained on deletion of the last 174 amino acids from the carboxy terminus of Mrs6p, i.e., the Mrs6$^{\Delta Xba}$ protein is unable to interact with the His-Ypt1p, whereas all others interact to different degrees.

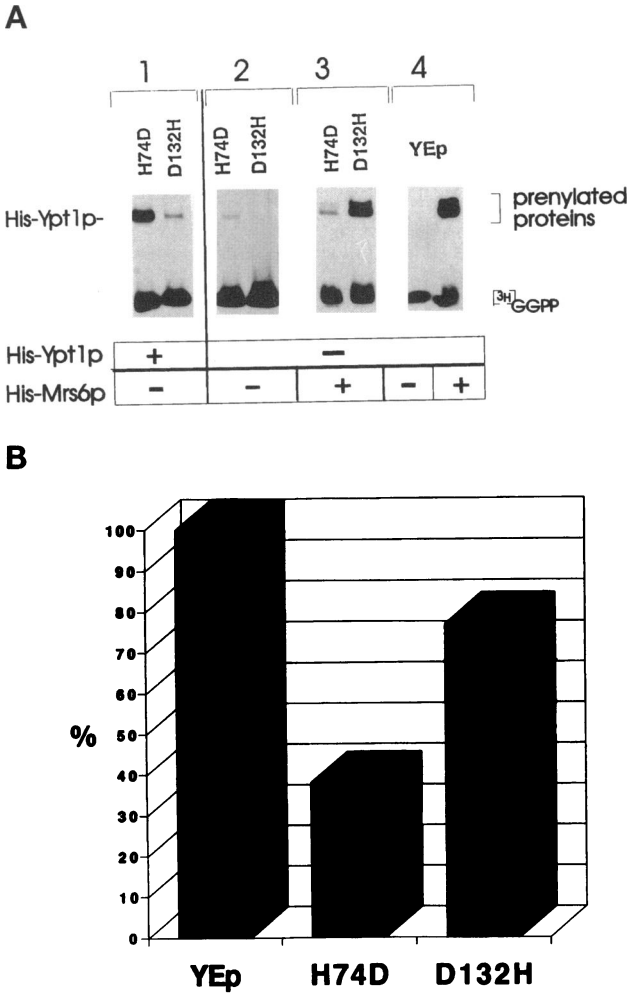

Figure 8. Unprenylated proteins accumulate in vivo in cells expressing the Mrs6^{H74D} and Mrs6^{D132H} proteins. (A) Autoradiogram of the geranylgeranylation assay performed as described in MATERIALS AND METHODS with (+) or without (−) the addition of His-Ypt1p or His-Mrs6p to crude extracts from Mrs6p-depleted cells expressing the Mrs6^{H74D} or Mrs6^{D132H} proteins or, as control, not expressing Mrs6p (YEp352-transformed cells). The positions of the labeled His-Ypt1 protein, the labeled endogenous proteins, or the unincorporated (^{3}H)GGPP are indicated. (B) Quantification of the prenylated proteins (from A, panels 3 and 4) shown as the average of two experiments (SD values: ±8% for H74D; ±4% for D132H). The amounts of prenylated proteins (in the range of 24–28 kDa) are indicated in percentages (%) relative to YEp351 (Mrs6p-depleted cells), which was taken as 100%.

To confirm that the carboxy-terminal deletions affect Ypt1p-binding and that the SCRs alone are not sufficient for such a function, we analyzed the ability of specific regions of the Mrs6p to interact with Ypt1p by the two-hybrid system. The two-hybrid system (Fields and Song, 1989) has been used extensively to show protein–protein interactions in many biological systems but also has proved to be a useful tool in detecting interactions between isolated domains of interact-

ing proteins (Allen *et al.*, 1995 and references therein; Espenshade *et al.*, 1995). To test whether the SCR motifs alone are sufficient for Ypt1p-binding by Mrs6p, we cloned a fragment encoding the first 322 amino acids of the Mrs6p (construct M6ΔBamHI, Figure 1A) in fusion with the Gal4-DNA–binding domain (M6Δ*Bam*HIpAS2 plasmid) and assayed it for its ability to bind the Ypt1 protein fused to the Gal4-activation domain (Ypt1pACTII plasmid). No interaction was observed by testing the β-galactosidase activity of the cotransformed cells either by a colony filter assay or more directly by measuring the activity in the crude extracts (Table 1). Because the fusion to the Gal4-DNA–binding domain of longer parts of the Mrs6p results in a high basal β-galactosidase activity, the DXba, DNhe, and PNS carboxy-terminal mutants were recloned in fusion with the Gal4-activation domain (pACTII constructs), whereas the Ypt1p was recloned in fusion with the Gal4-DNA-binding domain (pAS2 construct). In this orientation no basal activity was observed in cells transformed with the single plasmids (Table 1, right column). After cotransformation, β-galactosidase activity was tested by a filter assay and by measuring the enzymatic activity in crude extracts. Positive interactions were observed in cells cotransformed with the Ypt1pAS2 plasmid and the M6PNSpACTII or the M6ΔNhepACTII plasmids. No positive interactions were found by using the M6ΔXbapACTII plasmid.

These results (summarized in Table 1) essentially confirm the coimmunoprecipitation experiments showing that the SCRs alone do not contain all determinants for Ypt1p binding and that the region located between the *Xba*I (+429 aa) and the *Nhe*I (+570 aa) sites constitutes an essential part of the Ypt1-binding domain of the Mrs6 protein.

Table 1. The SCRs of Mrs6p do not contain all determinants required for Ypt1p binding as determined by the two-hybrid assay

		β-Galactosidase activity	
		YPT1	no YPT1
Mrs6ΔBamHI	(aa 1–322)	0.1 ± 0.02	0.2 ± 0.01
Mrs6ΔXba	(aa 1–429)	0.2 ± 0.01	0.3 ± 0.05
Mrs6ΔNhe	(aa 1–570)	13.8 ± 3.2	0.5 ± 0.01
Mrs6PNS	(aa 1–594)	12.3 ± 4.1	0.65 ± 0.1
no Mrs6		0.09 ± 0.01	*nd*

Y190 cells were transformed by plasmids carrying the Ypt1p or the different Mrs6p fragments in fusion either with the Gal4 DNA-binding domain (constructs pAS2) or with the Gal4 activation domain (pACTII), as described in the text. Units of β-galactosidase activity (nmol/mg × min) were calculated as (optical density at 420 nm × vol of assay)/(0.0045 × concentration of protein in extract assayed × time). Activities shown were the mean ± SD obtained from 10 different colonies tested. *nd*, not determined.

DISCUSSION

We provide a combination of genetic and biochemical evidence indicating that both the SCRs (Boguski and McCormick, 1993; Waldherr *et al.*, 1993b) and the carboxyl terminus of the yeast REP, the Mrs6 gene product, are involved in binding Ypt proteins. To analyze the functions of the RabGDI and REP common motifs, we mutagenized some of the most conserved residues in the SCR1a, SCR1b, SCR2, and SCR3b, introducing mutations that would alter the local charge without resulting in major perturbation of the predicted secondary structure of the Mrs6 protein. After the submission of this manuscript, the crystallographic structure and a mutational analysis of the bovine α-isoform of the RabGDI were published by Schalk *et al.*, 1996. This work has provided us with the possibility to compare our mutational analyses on the yeast REP with that performed in the corresponding regions of the bovine RabGDI. Studying the crystallographic structure of the bovine RabGDI, Schalk and colleagues observed that two major domains could be defined: domain I, containing the SCR1a, SCR1b, SCR3b, and the carboxy terminus of the protein that folds back toward the amino terminus; and domain II, constituted by an α-helical domain of ~100 amino acids that contains the SCR2 and the SCR3a. They also observed that all the SCRs are located on one side of the protein, which is therefore expected to be a highly conserved surface among the members of this family.

The mutations introduced into SCR1a, SCR1b, and SCR2 of the Mrs6p result in changes in the activity of Mrs6p, although to different degrees. The strongest effect was observed with mutation G53E in SCR1a. The glycine 53 of the Mrs6p is the first conserved glycine in the SCR1a motif, and it corresponds with glycine 11 of the bovine RabGDI, which is located between the first β-strand a1 and the α-helix A of domain I. This region is part of the GDI-CHM consensus domain formed by the SCR1 and the SCR3b. We have shown that the Mrs6^{G53E} protein is not able to sustain growth of Mrs6p-depleted cells, nor do these cells have any detectable RabGGTase activity. Moreover, at steady-state levels the Mrs6^{G53E} protein cannot be found in a complex with the endogenous Ypt1 protein. From this it is apparent that SCR1a of the Mrs6p is involved in the binding of Ypt proteins. Consistent with our results, Schalk and colleagues found that mutations in the SCR1a of the bovine RabGDI severely affect Rab3A binding and the RabGDI activity. Moreover, the fact that the Mrs6^{G53E} protein can form a complex with the histidine-tagged variant of the Ypt1 protein (compare Figure 7) but that the prenylation of this protein is blocked (compare Figure 5) suggests that glycine 53 or the structure of the region around this amino acid is important not only

for Ypt1p binding but also for the prenylation activity mediated by the Mrs6 protein (see below).

The mutational analyses that we have performed on the SCR1b and SCR2 show that mutations in these two motifs also alter substrate recognition. The mutant proteins Mrs6^{H74D} and Mrs6^{D132H} expressed in Mrs6p-depleted cells sustain growth, but the RabGGTase activity toward His-Ypt1p is moderately (H74D) or strongly (D132H) reduced. These proteins normally were found in a complex with the WT Ypt1 protein, but their ability to form a new complex with the unprenylated His-tagged Ypt1p is reduced to an extent comparable with their respective ability to prenylate this protein. Therefore, it seems that the Mrs6^{H74D} and Mrs6^{D132H} do not properly recognize His-Ypt1p. Because we have shown that Ypt proteins accumulate unprenylated in cells expressing the Mrs6^{H74D} or the Mrs6^{D132H} protein to an extent that correlates with the level of His-Ypt1p prenylation, we suggest that these mutant proteins have an altered binding ability not only toward the His-Ypt1p but also toward endogenous Ypt proteins. One explanation could be that the His-Ypt1p has slight structural changes that do not affect complex formation with the WT Mrs6p or the Mrs6^{Q289E} mutant protein but render its complex formation with the other Mrs6 mutant proteins more or less stable, depending on the nature of the mutation. In this case, because unprenylated proteins accumulate in the cells expressing the Mrs6^{H74D} or the Mrs6^{D132H}, we must assume that the binding defects observed with the His-Ypt1p also reflect an in vivo situation for which the types of experiments we have performed are not able to detect. An enhancement of the binding defects observed with the His-Ypt1p could be due to overloading the system with exogenous Ypt1 protein, which could shift the binding/releasing equilibrium of the Mrs6–Ypt1 protein complex toward one or other of the two forms of Ypt1p: the Mrs6^{G53E} (SCR1a), Mrs6$^{\Delta Nhe}$, and Mrs6^{PNS} proteins (carboxy terminus region) seem to have higher affinity for the His-Ypt1p, whereas the Mrs6^{H74D} and Mrs6^{D132H} proteins (SCR1b and SCR2, respectively) remain bound to the WT Ypt1p. Another explanation consistent with all data obtained with cells expressing the Mrs6^{H74D} or the Mrs6^{D132H} protein is that unprenylated proteins accumulate in cells because of an altered releasing constant of the REP/Ypt complex. A slow dissociation time with the Ypt substrate would likely result in a delay of the prenylation process without affecting the amount of the complex formed at steady-state levels but could affect the kinetics of the complex formation with the His-Ypt1p, because this protein is added in the assay together with the anti-Mrs6p antibody in the presence of the endogenous complex. A similar but opposite effect, e.g., fast dissociation time, could also explain why the Mrs6^{G53E}/His-Ypt1p complex is detectable while its instability would prevent prenylation of the His-Ypt1p substrate and the detection at steady-state levels of the complex formed with the endogenous Ypt1p.

We can therefore conclude that the mutations we have generated in the SCR1a, SCR1b, and SCR2 domains affect binding to Ypt1p. However, the clarification of the nature of these binding defects has to await more detailed analyses on the kinetics of complex formation. Here we show that the conserved glycine 53 (SCR1A box) and aspartic acid 132 (SCR2) of the Mrs6p seem to play prominent roles in binding Ypt proteins. Because no mutagenic analysis has been provided by Schalk and colleagues on the SCR2 box of the bovine RabGDI, nor, as in our case, have the in vitro binding studies been compared with the ability of the mutated RabGDI proteins to be able to substitute for the WT RabGDI form, for the moment we cannot draw other correlations except that the SCR1 box is clearly involved in Rab/Ypt binding in both REPs and RabGDIs.

The comparison of the data obtained with the mutation Q289E, located in the SCR3b, with the corresponding mutagenesis performed in the bovine RabGDI leaves some open questions. Mutations created in the bovine RabGDI in the SCR3b (E233S, R240A) lead to a >60-fold reduction in Rab3A binding, and <5% of Rab3A is extracted from membranes. The conserved glutamine in the SCR3b (Q289 in the Mrs6p, Q236 in the bovine RabGDI) is located between these two amino acids (G**E**xx**Q**GFx**R**xxAxxG), which form part of helix I of the SCR3b. However, we observe that the Q289E mutant protein can complement the Mrs6-depleted cells, RabGGTase activity is only slightly reduced (78% of the WT activity), and binding to the Ypt1 protein does not seem to be altered. The mutation we have introduced might not affect the Mrs6p activities analyzed in our work, or this particular amino acid in the SCR3b box has a minor role in Rab/Ypt binding.

Two-hybrid and protein deletion analyses have been used to investigate the domain structure of many proteins (Allen *et al.*, 1995 and references therein; Espenshade *et al.*, 1995; Nagiec *et al.*, 1995; Sanford *et al.*, 1995; de Mendez *et al.*, 1996). We have shown by these methods that, although the SCRs are necessary for Ypt1p binding, they are not sufficient alone.

In addition to the previously described conserved sequence motifs in RabGDI and Rab escort proteins, we report here on the presence of a further three motifs (RCR1–3), which are characteristic of the carboxy-terminal sequences of REPs alone. Deletions of the very last carboxy-terminal amino acids of the Mrs6 protein, but retention of the three REP-conserved carboxy-terminal motifs (located between amino acid 478 and amino acid 570), have very little effect on the Mrs6p activity: the Mrs6$^{\Delta Nhe}$ (deletion of 33 amino acids) and Mrs6^{PNS} (deletion of 9 amino acids) pro-

teins can complement the Mrs6p-depleted cells, and they have only slightly reduced RabGGTase activity and Ypt binding ability. The Mrs6$^{\Delta Xba}$ protein, which retains the SCRs but lacks the RCR motifs, is unable to sustain growth of the Mrs6 protein-depleted cells. Extracts from these cells lack RabGGTase activity caused by the inability of the Mrs6$^{\Delta Xba}$ protein to contact the Ypt small G proteins in vivo and in vitro, as shown by coimmunoprecipitation experiments and by the two-hybrid assay. Therefore, one of the regions essential for Ypt1 protein binding maps between amino acids 429 and 570 of the Mrs6 protein. These results are consistent with the observation that the carboxy-terminal region of the RabGDI participates in the stabilization of the GDI-CHM consensus domain, which is part of domain I. When the human and the yeast REP proteins are aligned with respect to the RabGDI/REP common motifs (SCR boxes), the deletion in the Mrs6$^{\Delta Xba}$ protein corresponds to the deletion expected at position 534 in the REP1 protein of a choroideremia patient having a frame-shift in the CHM gene (CHM2086; van den Hurk *et al.*, 1992). By analogy with the yeast system, patient CHM2086 may show low RabGGTase activity because of the inability of the REP1 protein to bind its Rab targets.

In conclusion, we have provided in vivo and in vitro data that show that Ypt1p-binding to the yeast REP protein depends on the contemporaneous presence of the SCRs and of the carboxy-terminal part of the protein. Further mutational analyses are in progress to better clarify the molecular structure of the yeast REP protein and to define other functionally important domains in this class of molecular chaperones.

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